Forest Research Report No. 110

# Frost Hardiness Testing: An Operational Manual for Use with Extended Greenhouse Culture

S.J. Colombo and C. Glerum

Ontario Tree Improvement and
Forest Biomass Institute
Ministry of Natural Resources
Maple, Ontario L0J 1E0
(416) 832-2761

and D.P. Webb

Canadian Forestry Service
Great Lakes Forest Research Centre
P.O. Box 490
Sault Ste. Marie, Ontario P6A 5M7
(705) 949-9461

Ontario Ministry of Natural Resources

Hon. Alan W. Pope
Minister

John R. Sloan
Deputy Minister

ISSN 0301-3924
ISBN 0-7743-9479-X

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Printed in Ontario, Canada

Additional copies of this publication
may be obtained from:

Ontario Tree Improvement and
Forest Biomass Institute
Ministry of Natural Resources
Maple, Ontario L0J 1E0
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Colombo, S.J., D.P. Webb and C. Glerum. 1984. Frost hardiness testing: An operational manual for use with Extended Greenhouse Culture. Ontario Ministry of Natural Resources, Forest Research Report No. 110. 14 p.

## ABSTRACT

The procedures and equipment necessary for monitoring the frost hardiness of container-grown seedlings in preparation for overwintering outside are described. Procedures for sample collection, freezing tests and estimation of freezing injury are outlined for routine monitoring of large numbers of samples. Guidelines, based on monitoring results over a number of developmental years, are presented to aid nurserymen in the use of frost hardiness tests to guide the *Extended Greenhouse Culture* method of hardening spruce container seedlings for overwintering.

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## INTRODUCTION

In Ontario, over 25 million container tree seedlings were produced in 1983, which accounted for approximately 25% of all nursery stock produced. Almost half of this stock is grown in greenhouses during the summer and overwintered out-of-doors for planting the following spring. It is during overwintering that container seedlings, especially spruce, are most likely to suffer damage.

Overwinter losses of up to 50% have been observed in some black spruce (Picea mariana (Mill.) B.S.P.) crops since monitoring began in 1981 (Colombo 1982). Seedlings suffered severe overwinter damage when they were moved outside and exposed to frost before significant hardening had taken place. In comparison, seedlings which were left inside the greenhouse to develop high levels of frost hardiness under short photoperiods and warm temperatures suffered little or no overwinter damage.

A technique called *Extended Greenhouse Culture* (Colombo et al. 1982a,b) has recently been introduced in Ontario for hardening spruce container seedlings. *Extended Greenhouse Culture* relies upon monitoring of frost hardiness during the fall to determine when seedlings may be safely moved out-of-doors. Procedures developed to perform frost hardiness tests using the electrolytic method are described in this report. The electrolytic method, which is being used operationally in Ontario to measure damage caused in controlled freezing tests, provides rapid results and a quantitative measure of the degree of frost hardiness of tree seedlings (Wilner 1962, Aronsson and Eliasson 1970, van den Driessche 1976, Green and Warrington 1978, Colombo et al. 1982b). To date, *Extended Greenhouse Culture* has been used extensively to harden black and white spruce container seedlings. Preliminary results using jack pine have also been favourable. In 1984-1985 in Ontario over 20 million seedlings have been prepared for overwintering using *Extended Greenhouse Culture*, and regional laboratories for frost hardiness testing are located in Swastika and Dryden. Through the application of the freezing test methods outlined in this report, used in conjunction with *Extended Greenhouse Culture*, it is hoped that the quality of overwintered container seedlings produced in this province will be increased. This manual is intended for use by nurserymen for testing the state of frost hardiness in container seedling crops being hardened for overwintering using *Extended Greenhouse Culture*.

## PROCEDURES

The electrolytic method makes use of electrical conductivity measurements to determine the extent of damage to seedling shoots caused in a controlled freezing test. Seedling shoots are frozen, then placed in water, after which the water's electrical conductivity is measured. If there is a large increase in the electrical conductivity of the water, freezing damage will have been severe, indicating a low level of frost hardiness. When, however, electrical conductivity remains unchanged after freezing at low temperatures, little or no damage will have occurred and frost hardiness will be high. It is then considered safe to end the period of *Extended Greenhouse Culture* and to move the stock outside.

### Equipment Required

The equipment being used to perform freezing tests and measure damage using the electrolytic method is: 1/ a freezer capable of reaching -40°C at

a controlled rate of freezing, 2/ a remote temperature probe to monitor sample temperature, and 3/ a conductivity meter. Total cost to set up a laboratory for performing freezing tests is approximately $20 000.

A freezer specially designed to reach -40°C is recommended because household freezers are inadequate below -20°C . As the results of freezing tests can be affected by how quickly the temperature of the sample decreases, it is necessary to control the rate of freezing. Our testing is standardized at a freezing rate of 5°C per hour. This rate of freezing can be achieved by placing an insulated box containing the samples into a freezer set at a constant low temperature, or by attaching a programmable temperature controller to the freezer. For reproducibility the use of a programmable temperature controller is preferred. Several models of programmable freezers are available. We have used a 0.33 $m^3$ (12 cu.ft.) chest freezer from Foster Refrigeration Ltd.[1], (Model No. LT-12-E-40) (Fig. 1), to which a Honeywell[1] cam temperature programmer (Fig. 2) (Model No. AR52ADDO-056) and an air circulation fan were attached by Constant Temperature Control Ltd.[1] (approximate cost $10 000.).

Temperature of the samples must be measured to determine when the shoot tissues reach the designated test temperatures. As temperature variations between sample bottles may occur, it is best to monitor the temperature of several bottles simultaneously. Therefore, a multipoint temperature recorder using thermocouples is recommended for use. Cost of a 24-channel recorder ranges from $5 000 to $6 000. Alternatively, temperature may be monitored manually by use of a thermocouple thermometer, which ranges in price from about $300 to $1 000.

A conductivity meter with digital readout and autoranging features allows rapid measurement of large numbers of samples. The Radiometer[1] Model CDM83 conductivity meter provides these features. The cost of this and comparable instruments is about $3,000. In addition to these major pieces of equipment, jars are required to hold tree seedling samples during testing. We recommend 125 ml glass jars which have a wide mouth (4 cm) and watertight screw cap. For freezing tests the jars are placed to a depth of about 4 cm in holes cut in a 5 cm thick sheet of rigid foam insulation, which reduces temperature fluctuations inside the jars during freezing.

## Monitoring Schedule

It is recommended that frost hardiness testing be done in two stages. The first stage monitors the transition from non-frost hardy to hardy seedlings. Samples from only one greenhouse per nursery are needed, beginning at the time 100% of the seedlings have initiated buds, and sampling should be continued on a weekly basis until significant increases in frost hardiness have occurred. At that time, the second stage of monitoring should begin.

The second stage of frost hardiness testing is used to determine when stock becomes sufficiently hardy to withstand frosts when transferred outside for overwintering. The hardiness level of seedlings in each greenhouse is determined on at least a weekly basis. The criteria used to assess safe dates for moving seedlings outside and procedures for running freezing tests are described later in this report.

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[1]Mention of company or brand names does not signify recommendation or endorsement.

Figure 1. Freezing unit for performing controlled freezing tests

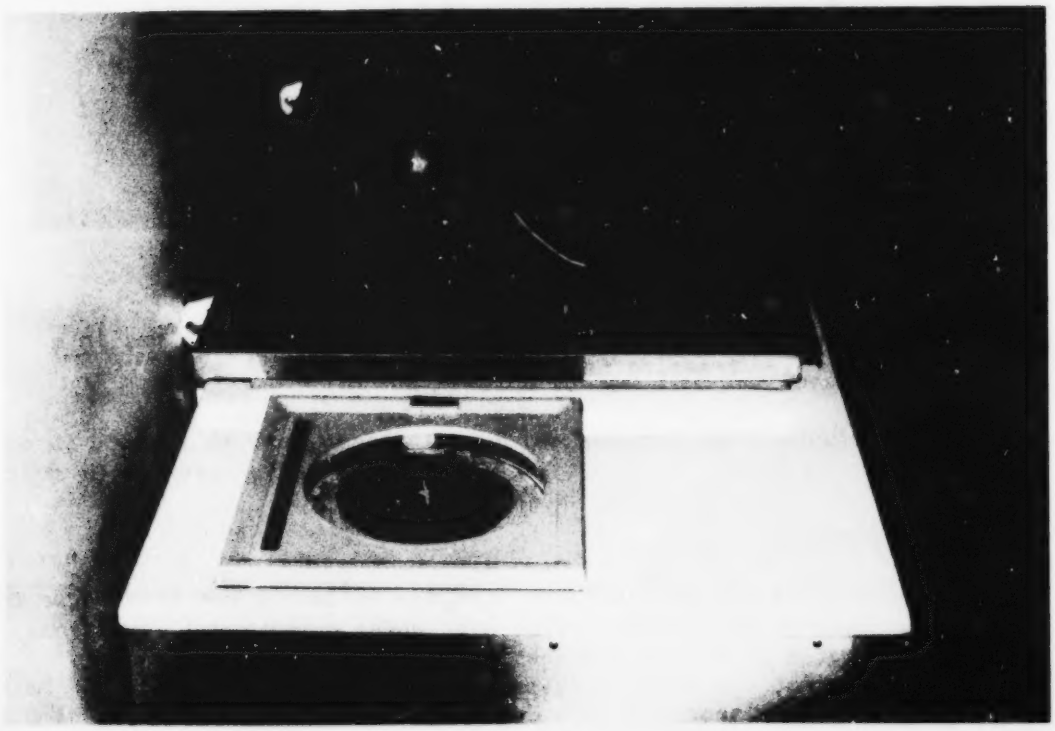

Figure 2. Cam temperature controller attached to freezing unit to lower temperature at controlled rates

## The Sample Material

The terminal 2-3 cm of the shoot is collected using sharp scissors or secateurs (Fig. 3). Shoot tips are sealed in a plastic bag and placed in a refrigerator (approximately 5°C) until use. Do not allow the samples to freeze. Samples have been stored for up to three days in this manner without measurable changes in frost hardiness.

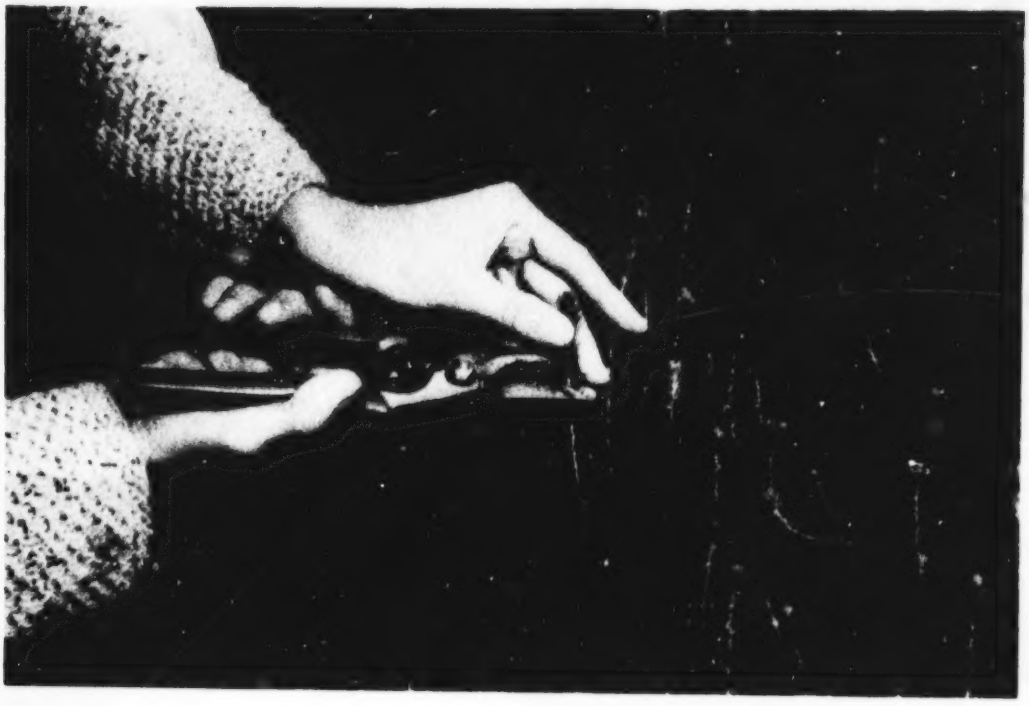

Figure 3. Black spruce seedling shoot tip being severed for use in a freezing test

## Sample Collection

For each freezing test 45 shoot tips are collected per greenhouse over as large an area as can reasonably be done under operational conditions. Reliable estimates of frost hardiness have been obtained by randomly selecting one shoot tip from every second or third tray sampled down both sides of greenhouses accessed by a central aisle. Seedlings from which the shoot tip has been collected can remain in the tray, as shoot elongation in the second year will not be greatly reduced as a result of decapitation.

## The Freezing Test

To determine the amount of damage caused as a result of freezing, it is necessary to use 15 of the 45 collected shoot tips as a non-frozen control, leaving the remaining 30 shoot tips (two replicates of 15) for freezing. These methods are summarized in Appendix 1, with a sample form for recording test results in Appendix 2.

Control Samples - Place the 15 control shoot tips in one sample jar, and rinse them with distilled or deionized water. Discard the rinsing water and fill the jar with distilled or deionized water a second time, so that all of the shoot tips are completely immersed. In the jars we are using, 90 ml of water is sufficient. A piece of aluminum foil can be used to hold down any shoot tips which may float on the surface of the water. Cap the jar tightly and leave overnight at room temperature (20-25°C). The following morning shake the jar vigorously by hand (eg. shaken 20 times by hand in 10 seconds). Following shaking, the electrical conductivity is measured immediately and recorded. This is the "Control" electrical conductivity value (EC control).

To determine the total electrolytic content of the control shoot tips, the tissues are killed by placing the tightly-capped jar (shoot tips and water) into an oven (90°C) for two hours. After killing, place the sample at room temperature overnight so that the released electrolytes can enter solution. Then, shake the still-sealed bottle vigorously and measure the electrical conductivity of the killed tissues (EC killed control). The electrical conductivity values for control and killed control are used to calculate the relative conductivity of the control samples (RC control) as follows:

$$\text{RC control} = \frac{\text{EC control}}{\text{EC killed control}} \times 100$$

where RC control = relative conductivity of the control samples;

EC control = electrical conductivity of the water in which non-frozen shoots were immersed <u>before</u> killing;

and EC killed control = electrical conductivity of the water in which non-frozen shoots were immersed <u>after</u> killing.

The RC control is that proportion of the shoot's total electrolytic content which is released without freezing.

Frozen Samples - Place the 30 shoot tips to be frozen in one appropriately labelled jar. Rinse the shoot tips once with distilled or deionized water, discard the rinsing water, and cap the jar tightly. Place the jar into a sheet of rigid foam insulation (Fig. 4) and put the sheet into the freezing unit (Fig. 5). Run the freezing unit at 5°C for one hour to allow the sample temperature to equilibrate. After equilibration, the temperature is decreased at a rate of 5°C per hour to the desired minimum test temperature. A minimum test temperature of -10°C is used for non-hardy tissue, and as frost hardiness develops minimum temperatures of -15°C to -30°C can be used. At present we test at both -10 and -15°C throughout the monitoring schedule.

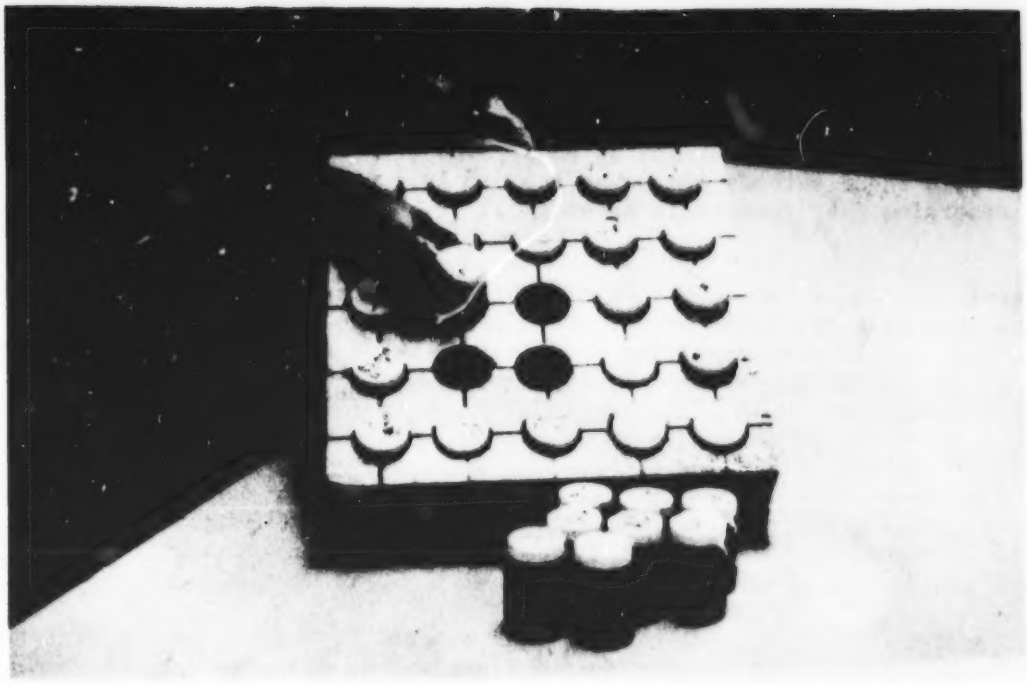

Figure 4. Jars placed in a sheet of rigid foam insulation in preparation for placement in the freezing unit

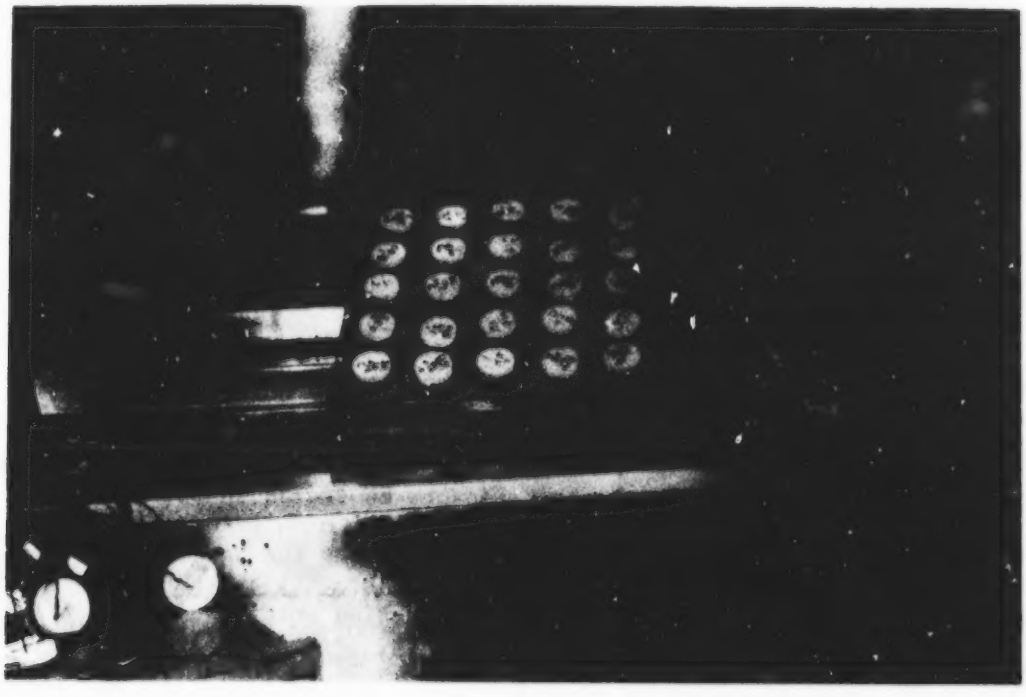

Figure 5. Sheet containing bottles of shoot tips placed in the freezing unit

When the test temperature has been reached the styrofoam sheet with samples is taken out of the freezer, covered with an insulating foam rubber sheet (Fig. 6) and placed in a cooler to allow slow thawing overnight. The next day the sample is split into two replicates of 15 shoot tips each, and distilled or deionized water is added to the jar to completely cover the shoot tips. The tightly capped jar is then left overnight to allow electrolytes to diffuse into the water from the previously frozen shoot tips.

Figure 6. Foam rubber insulation placed over bottles after freezing to allow slow thawing

The following morning shake the jar vigorously before measuring the electrical conductivity of the water holding the previously frozen shoot tips (EC frozen). Kill the previously frozen shoot tips by placing the jar (including water) into a 90°C oven for two hours. Let stand overnight at room temperature (20-25°C) to allow the leaching of electrolytes from the shoots. Shake the jar vigorously and measure electrical conductivity (EC killed frozen). The proportion of the total shoot electrolytes released into the water due to freezing induced damage (RC frozen), is calculated as follows:

$$\text{RC frozen} = \frac{\text{EC frozen}}{\text{EC killed frozen}} \times 100$$

where: EC frozen = electrical conductivity of the water in which frozen shoots were immersed before killing

and, EC killed frozen = electrical conductivity of the water in which frozen shoots were immersed after killing.

## Estimating Frost Hardiness

Frost hardiness is expressed using an Index of Injury ($I_t$), calculated on a percentage basis (Flint *et al.* 1967):

$$I_t = \frac{\text{RC frozen} - \text{RC control}}{1 - \frac{\text{RC control}}{100}}$$

The Index of Injury is an expression of the amount of damage induced by freezing. When the Index of Injury has a high value, frost hardiness will be low. Low Index of Injury values indicate high levels of frost hardiness. A summary of calculations for relative conductivity and Index of Injury are given in Appendix 3. The Index of Injury values are independent of sample volume and of seasonal variations in the quantity of "free" electrolytes in the shoot which are released without freezing.

When establishing a new laboratory for frost hardiness testing, we have found it necessary to have a period of pre-testing before operational frost hardiness testing can begin. During pre-testing the levels of Index of Injury for the new laboratory for non-frost hardy and hardy seedling shoots are determined, as these levels can differ widely between laboratories.

## Choosing Test Temperatures

In the first stages of testing it is necessary to use a freezing temperature which is moderate in severity. A proper temperature will badly damage the initial, non-hardy shoot tips tested, and, as frost hardiness increases, the amount of damage induced will decrease. We have found that temperatures of -10° and -15°C allow the initial stages of frost hardening to be detected. Temperatures of -20°C and lower cause severe damage to shoot tips until relatively high levels of frost hardiness are reached, thus obscuring the beginning of frost hardiness development.

## FROST HARDINESS MONITORING AS PART OF EXTENDED GREENHOUSE CULTURE

The purpose of performing frost hardiness tests is to determine when container seedlings may be safely moved outside for overwintering. *Extended Greenhouse Culture* is a reliable method of hardening spruce container seedlings for overwintering because it uses the objective information provided from frost hardiness testing to decide when to remove stock from the greenhouse (Colombo 1982). In the past, hardening for overwintering was attempted by following a brief (two to three week) schedule, usually in August, of reduced nitrogen fertilization and greenhouse cooling. This system of hardening seedlings has often been inadequate because it did not account for the time needed to pass from a state of active height growth to bud initiation and the completion of bud development. We have found that high levels of frost hardiness in black and white spruce seedlings are achieved following the completion of bud development.

The *Extended Greenhouse Culture* system uses both bud dissections (to determine degree of development) and frost hardiness tests to provide the nurseryman with information he can use to make operational decisions. A typical example of the results of frost hardiness and bud development monitoring of black spruce in northern Ontario is shown in Figure 7. Frost hardiness increases and decreases during the course of hardening in the fall. As shown on August 30 and November 1, negative $I_t$ values are

possible. Also, in some instances (August 22, October 11 and November 8), seedlings frozen to -10°C can have higher Index of Injury values than occur at -15°C. At the time of early bud initiation ("A" in Fig. 7) when height growth has nearly been completed there is a temporary increase in frost hardiness. As the rate of needle primordia initiation in the bud reaches a maximum, the level of hardiness decreases. When bud development is complete ("B" in Fig. 7) high levels of frost hardiness are maintained. Seedlings would have been considered ready to be moved outside October 25.

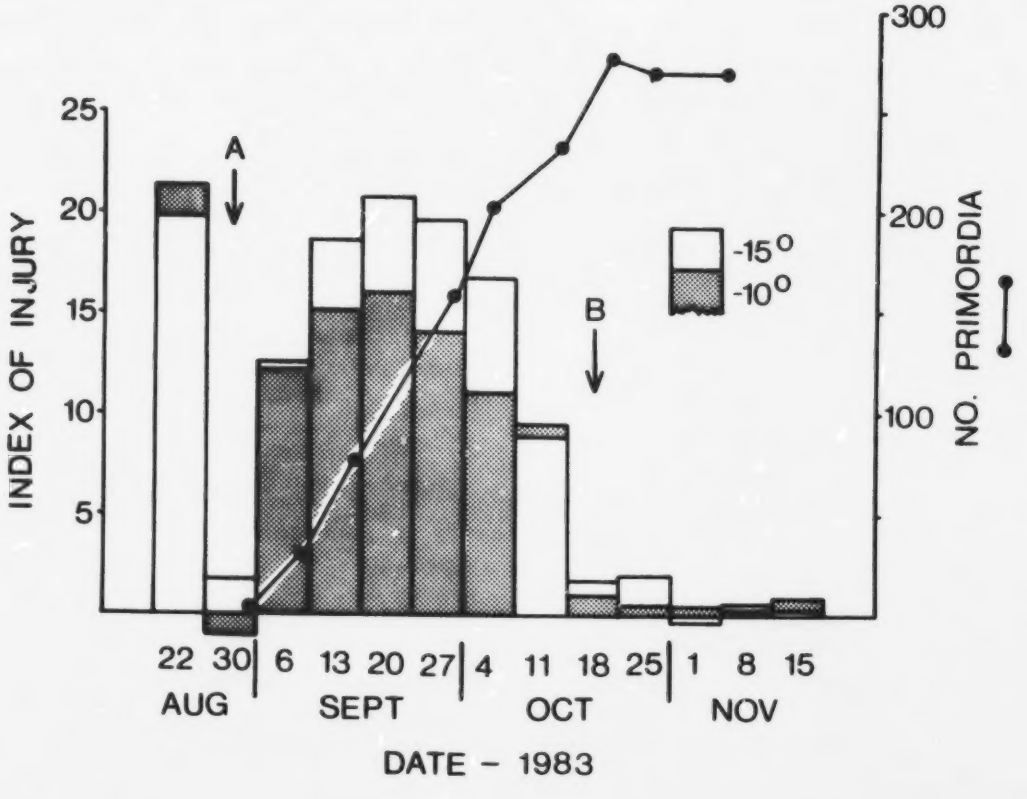


Figure 7. A typical pattern of frost hardiness and bud development for black spruce container seedlings in northern Ontario

## RECOMMENDED HARDENING REGIME FOR SPRUCE CONTAINER STOCK

Based on our observations of the effect of greenhouse procedures on the onset of frost hardiness, the following *Extended Greenhouse Culture* hardening regime is currently recommended for operational use in Ontario:

1) Stop fertilization and leach growing medium when seedlings are within 3 cm of desired height, or no later than August 31. Use short (8 h) day treatment to initiate buds if possible. Begin frost hardiness monitoring of seedlings in one greenhouse at each nursery. Dissect shoot tips to determine terminal bud initiation in all greenhouses at all nurseries.

2) Apply water only (no fertilizer) and maintain optimum growing temperatures (20 - 30°C) until 100% of the seedlings in each greenhouse have initiated buds (determined by dissecting a sample of 20 shoot tips weekly).

3) Following 100% bud initiation, resume fertilization at normal rates for optimum growth (100 - 200 ppm nitrogen using a 20-20-20 fertilizer). Terminate short day treatment if used. Continue bud dissections and freezing tests in one greenhouse at each nursery. Maintain optimum growing temperatures until bud development (as shown by numbers of needle primordia) nears completion in the test greenhouse.

4) Following the completion of bud development, and when higher levels of frost hardiness are achieved in the test greenhouse, begin frost hardiness testing of seedlings in all greenhouses. Commence continuous cooling of each greenhouse (-2°C to +5°C) when seedlings from that greenhouse show increased levels of frost hardiness.

5) Based upon frost hardiness testing of each greenhouse, move crops outside when there have been two successive weeks of high levels of frost hardiness at both -10 and -15°C.

## ACKNOWLEDGEMENTS

The enthusiastic support for the *Extended Greenhouse Culture* program by the Ministry's Northern Region staff, in particular that of R. Cameron, is gratefully acknowledged.

This manual is the result of a project that was jointly funded by the Governments of Canada and Ontario under the Forest Management Subsidiary Agreement (Project No. 585042).

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## APPENDIX I. Summary of methods for measuring frost hardiness by the electrolytic method.

Day 1 — Collect 45 shoot tips (2-3 cm long) from one greenhouse. Rinse all shoots in distilled water. Discard rinse water. Subdivide the shoot tips into a control and frozen samples.

a) Control

Day 1
- i) Place 15 shoot tips in one jar to serve as the control.
- ii) Add distilled or deionized water to cover the shoot tips.
- iii) Seal the lid on the jar and leave overnight.

Day 2
- i) Shake the jar containing control shoot tips.
- ii) Measure electrical conductivity of the water in the jar.
- iii) Place the tighly capped jar in a 90°C oven for two hours to kill the shoot tips.
- iv) Remove jar from the oven and leave overnight.

Day 3
- i) Shake the jar containing the killed control shoot tips.
- ii) Measure electrical conductivity of water in the jar (EC killed control).
- iii) Discard control samples.

b) Frozen

Day 1
- i) Seal 30 shoot tips in one jar and put the jar in the freezing unit.
- ii) Expose the shoot tips in the jar to the selected minimum temperature.
- iii) Remove the jar of frozen shoot tips from the freezing unit and place in a cooler for slow thawing.

Day 2
- i) Subdivide the sample of 30 frozen shoot tips into two replicates of 15 tips in two separate jars.
- ii) Add distilled or deionized water to cover the previously frozen shoot tips.
- iii) Leave overnight.

Day 3
- i) Shake the jars containing previously frozen shoot tips.
- ii) Measure electrical conductivity of the water in the jars (EC frozen).
- iii) Place tightly capped jars in a 90°C oven for 2 hours to kill the shoot tips.
- iv) Remove jars from the oven and leave overnight.

Day 4
- i) Shake the jars containing the frozen and killed shoot tips.
- ii) Measure electrical conductivity (EC killed frozen).
- iii) Discard samples.
- iv) Using electrical conductivity values, calculate the Index of Injury (Appendix III).

## APPENDIX II. FROST HARDINESS TEST - DATA FORM

NURSERY:_____ GREENHOUSE NO.:_____ SPECIES:_____

| Test Date 19__ | Test Temp (°C) | Control | | | Frozen | | | | | | | | Average Index of Injury % $\frac{I_{t1}+I_{t2}}{2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Conductivity | | Rel. Cond. (%) | Rep. #1 | | | | Rep. #2 | | | | |
| | | | | | Conductivity | | Rel. Cond. (%) | $I_t$ (%) | Conductivity | | Rel. Cond. (%) | $I_t$ (%) | |
| | | control | killed control | | frozen | killed frozen | | | frozen | killed frozen | | | |
| | | | | | | | | | | | | | |

## APPENDIX III. Calculations for determining Index of Injury

$$\text{RC control (\%)} = \frac{\text{EC control}}{\text{EC killed control}} \times 100$$

$$\text{RC frozen (\%)} = \frac{\text{EC frozen}}{\text{EC killed frozen}} \times 100$$

$$I_t \text{ (\%)} = \frac{\text{RC frozen} - \text{RC control}}{1 - \frac{\text{RC control}}{100}}$$

where EC = electrical conductivity of control (not frozen), frozen, and killed shoot tips.

RC = relative conductivity

and $I_t$ = Index of Injury